DETECTION OF GRAM POSITIVE AND GRAM NEGATIVE ORGANISMS IN SPUTUM QUALITY TESTING

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ABSTRACT

Sputum is a mucus that cough up from the lower airways which is a normal body fluid. The sputum consists of squamous epithelial cell, pus cells and bacteria. For this project, it only focus on bacteria organisms which is consists of two type of bacteria such as gram positive and gram negative bacteria. The purpose of this project is to detect and count the quantity for gram positive and gram negative bacteria. At the same time, the grading for both bacteria is identified based on grading criteria. Currently, gram positive and gram negative bacteria is detected and counted manually by human and the grading is identified. Since human might do some mistake in detection and summation for both bacteria and take a long time in doing this process, developing an automatic vision system is necessary to obtain more accurate results and time saving. This automatic vision system developed based on image processing technique which is involve of software simulation only by using MATLAB simulation. In developing this project, some techniques of the image processing is applied into MATLAB simulation such as image analysis, image segmentation, image enhancement, morphological process and other. Then, the results for summation and grading are displayed on MATLAB Graphical user Interface (GUI). Last but not least, the result for grading obtained give similar value compare to validation test from HUSM.

ABSTRAK

Kahak merupakan lendir yang keluar daripada saluran udara yang lebih rendah ketika batuk dan ia merupakan bendalir badan yang normal. Kahak terdiri daripada *squamous epithelial cell, pus cell* dan bakteria. Untuk projek ini, ia hanya memberi tumpuan kepada organisma bakteria yang terdiri daripada dua jenis bakteria iaitu gram positif dan gram negatif. Tujuan projek ini adalah untuk mengesan dan mengira kuantiti untuk gram positif dan gram negatif. Pada masa yang sama, penggredan bagi kedua-dua bakteria dikenalpasti berdasarkan kriteria penggredan. Pada masa kini, bakteria gram positif dan gram negatif dikesan dan dikira secara manual oleh manusia dan penggredan itu dikenalpasti. Memandangkan manusia mungkin melakukan kesilapan semasa mengesan dan mengira untuk kedua-dua bakteria dan mengambil masa yang panjang dalam melakukan proses ini, membangunkan *automatic vision system* adalah perlu untuk memperolehi keputusan yang lebih tepat dan menjimatkan masa. *Automatic vision system* yang dibangunkan berdasarkan teknik pemprosesan imej yang hanya melibatkan simulasi perisian dengan menggunakan simulasi MATLAB. Dalam membangunkan projek ini, beberapa teknik pemprosesan imej dilaksanakan ke dalam simulasi MATLAB seperti analisis imej, segmentasi imej, peningkatan imej, proses morfologi dan lain-lain. Kemudian, keputusan untuk penjumlahan dan penggredan dipaparkan pada MATLAB *Graphical User Interface (GUI)*. Akhir sekali, hasil untuk penggredan yang diperolehi memberi hasil yang hampir sama berbanding dengan ujian pengesahan dari HUSM.

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Sputum is a mucus that produced in the lungs and in the lower airways leading to the lungs. It is a normal body fluid, though excessive amounts of sputum often signal pulmonary disease. The colour consistency of sputum provide clues about the health of lungs and airways, though are not reliable diagnostic characteristics by themselves.

This project will focus on detection and counting the quantity of gram positive and gram negative in sputum sample. The image of sputum samples are taken under x100 computerized microscope which are obtained from Hospital Universiti Sains Malaysia (HUSM). Since the human might do some mistake in detection and summation for both bacteria and take a long time in doing this process, developing an automatic vision system based on image processing technique is necessary to obtain more precious results and save the time. Some techniques of the image processing will be used such as image analysis, image segmentation, image enhancement, morphological process and also the other techniques.

This vision system simulation is develop using MATLAB R2010a. All the process of detection and summation will be simulate by MATLAB simulation. Last but not least, the results for summation and grading will be displayed on MATLAB Graphical user Interface (GUI).

1.2 Problem Statement

The process of detection and summation for gram positive and gram negative bacteria in sputum sample nowadays is manually done by human. Since the human might do some mistakes in bacteria detection and summation, and taking a long time in doing this process, an automatic vision system is needed to be developed to avoid that problem occurs. Besides, this automatic system also will obtain more accurate results than previous method which is manually done by human. The grading of the bacteria in the system are calculated properly instead of the normal practice which are just by assumption.

1.3 Objective

The objectives of this project are:

i. To develop the vision system which is able to detect and count the quantity of the gram positive and gram negative bacteria in sputum sample image.
ii. To identify the grading of quantity for gram positive and gram negative bacteria in sputum sample image.

1.4 Scope of Project

This project involves software development only which is the vision system development. This system able to detect and count the quantity of gram positive and gram negative in sputum sample. Various techniques of image processing used for this system which can be applied by using MATLAB R2010a. The image taken from digital microscope under x100 magnification is processed to get the image of sputum which is contains variety bacteria including unwanted objects. So, to obtain the image needed, the associated coding is written on M-File. Then, the final result is

shown through the MATLAB GUI system which is display the number and the grading of gram positive and gram negative based on the final image.

CHAPTER 2

LITERATURE REVIEW

The related literature reviews of this project which have been referred according to this project's needs will be explained in this chapter. Most required literature reviews are focused on the introduction and differences both bacteria, techniques in image processing and how to apply those techniques in this project by using MATLAB simulation.

2.1 Differences between Gram Positive and Gram Negative bacteria

The differences for both bacteria can be determined based on their morphology such as cell shape and size. For cell shape, there are three main shapes which are determined by the molecular properties of cell wall such as spherical, rod-like, spiral and curved forms while pleomorphic form have no defined shape because it lacked a rigid cell wall. Meanwhile, the most bacteria have size in the range of 1-5μ in length [1].

2.1.1 Gram Positive bacteria

Gram positive bacteria have a thick multilayered, peptidoglycan cell wall that is exterior to the membrane. The peptidoglycan in most gram positive bacteria is covalently linked to teichoic acid, which is essentially a polymer of substituted glycerol units linked by phosphodiester bonds. All gram positive bacteria also have teichoic acid in their membranes, where it is covalently linked to glycolipid. The teichoic acids are major cells surface antigens [1]. The cell wall structure and example of gram positive will be shown in Figure 2.1 and Figure 2.2.

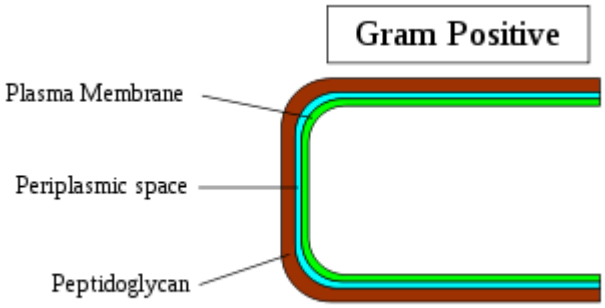


Figure 2.1 Gram positive cell wall structure

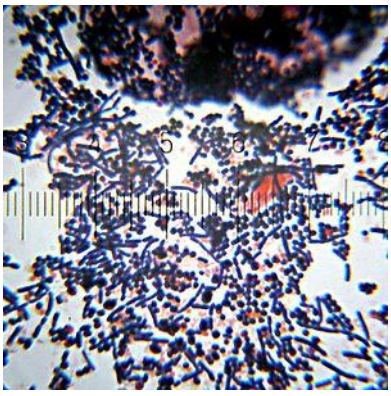


Figure 2.2 Gram-positive bacteria, stained purple, of both the bacillus ("rod-shaped") and cocci (spherical) forms. A few Gram-negative bacteria are also present, stained pink.

2.1.2 Gram Negative bacteria

Gram negative bacteria have two membranes – an outer membrane and inner (cytoplasmic) membrane. Their peptidoglycan layer is located between the two membranes in what is called the periplasmic space. The periplasmic space also contains enzymes and various other substances. In contrast to gram positive cells, the peptidoglycan layer of gram negative is thin, and the cells are consequently more susceptible to physical damage. The outer membrane is distinguished by the presence of various embedded lipopolysaccharides. The polysaccharide portion (O-polysaccharide) is antigenic, and can therefore be used to identity different strains and species. The lipid portion (lipid A) is toxic to humans and animals. Lipid A, because it is an integral part of membrane, is called an endotoxin, as opposed to exotoxins, which are secreted substances [1]. The cell wall structure and the example of gram negative will be shown in Figure 2.3 and figure 2.4.

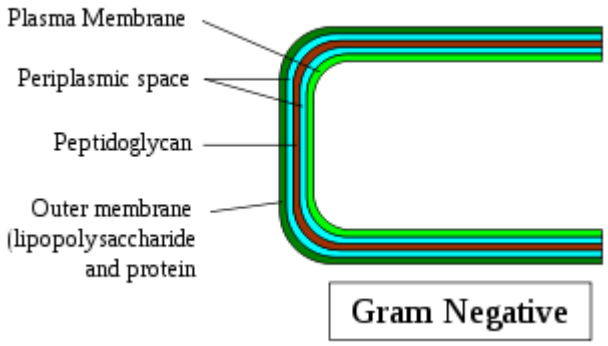


Figure 2.3 Gram negative cell wall structure

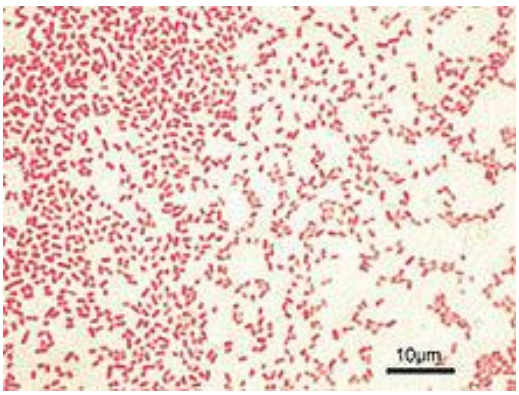


Figure 2.4 Microscopic image of Gram-negative Pseudomonas aeruginosa bacteria (pink-red rods)

Figure 2.5 shows the sputum sample image taken under x10 magnification using digital microscope that used for detection and summation of pus cell and squamous epithelial cells.

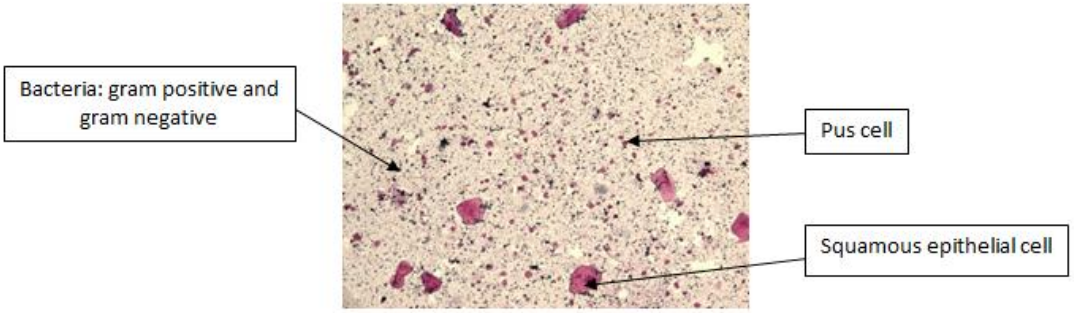


Figure 2.5 Sputum image under x10 computerized microscope

Figure 2.6 shows the sputum sample image taken under x100 magnification using digital microscope that used for detection and summation of bacteria (gram positive and gram negative).

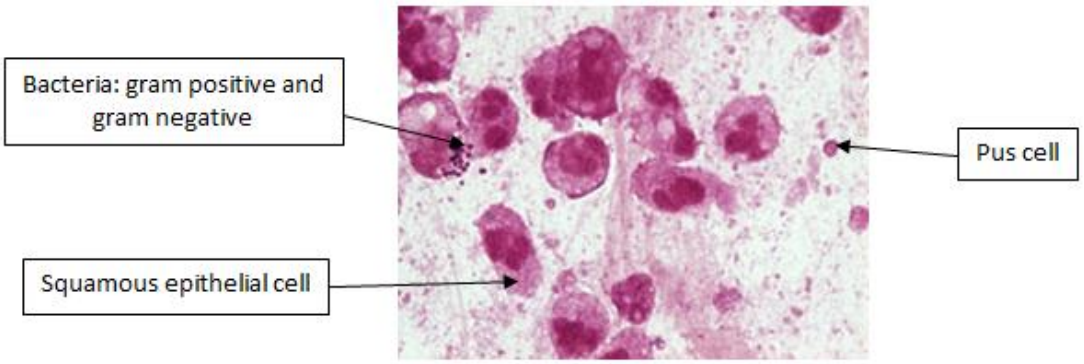


Figure 2.6 Sputum image under x100 computerized microscope

2.2 Staining Properties

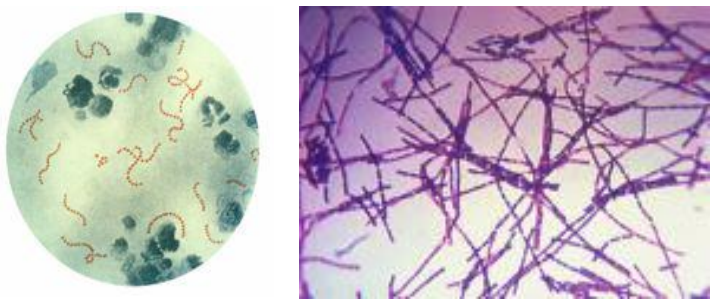
The staining properties are used to differentiate both bacteria through gram stain and counterstain process

2.2.1 Gram Stain

The gram stain is used to classify bacteria on the basis of their forms, sizes, cellular morphologies, and gram reactions. Besides, it is additionally a critical test for the presumptive diagnosis of infectious agents and serves to assess the quality of clinical specimens [2]. In this situation, gram stain is a quick procedure used to look for the presence of bacteria in tissue samples and to characterize bacteria as gram positive or gram negative, based on the chemical and physical of their cell walls [1].

i. Get sputum sample for gram stain.
ii. Add 1 or 2 drop of sputum sample onto glass slide.
iii. Heat fix the smear, by quickly passing it two three times through a flame, or heat it on top of an electric slide warmer.
iv. Flood the smear with crystal violet solution; allow to acts for 1 minute.
v. Rinse the slide, the flood with iodine solution and allow iodine to act for 1 minute. All organisms appear purple, that are gram positive.
vi. Rinse off excess iodine. Decolorize with acetone, approximately 5 seconds (time depends on density of specimen).
vii. Wash slide immediately with water. After acetone decolorization, those organisms that are gram negative are no longer visible.

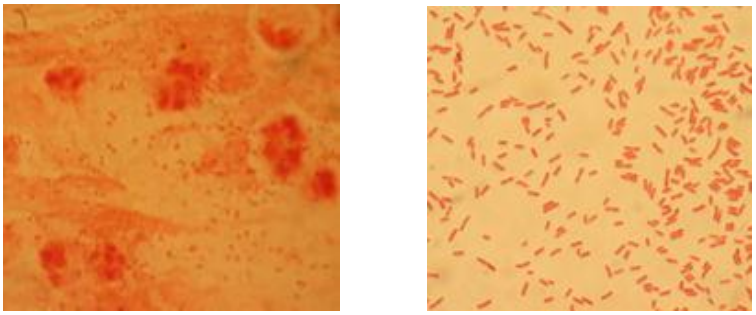
Figure 2.7 shows the examples of gram positive bacteria after doing the gram staining process.



(a) (b)

Figure 2.7 (a) Gram-positive (purple) cocci (round cells) in chains (b) Gram-positive rods

Figure 2.8 shows the examples of gram negative after doing the gram staining process.



(a) (b)

Figure 2.8 (a) Gram-negative diplococci (pink, spherical bacteria appearing as pairs), both inside and outside cells (b) Gram negative rod

2.2.2 Counterstains

In the gram staining procedures, the bacteria cells may be rendered invisible by the decolorization step. Visibility can be restored by using a counterstain that has a color distinctly different from the primary stain. The pink dye, safranin is used in the counterstain procedure. Therefore, gram positive cells are purple (having retained the crystal violet) whereas gram negative cells are pink (having been counterstained with safranin) [1].

i. Apply safranin counterstain for 30 seconds.
ii. Wash in water, blot and dry in air. Gram negative organisms are visualized after the application of the counterstain.

2.3 The Grading of Microorganisms

The grading of microorganisms as shown in Table 2.1 which is used in grading for gram positive and gram negative based on the quantity for both bacteria in a sputum sample.

Table 2.1 The grading of Microorganisms [8]

Occasional	Very few seen
1+	1-5 cells per field / a quarter of the field
2+	5-10 cells per field / half of the field
3+	10-25 cells per field / three quarters of the field
>25 to Numerous	Packed field / the whole field

Table 2.2 shows the example of result based on type of gram positive and gram negative in sputum sample.

Table 2.2 The reported result for bacteria component [16]

Reported results	A Labs	B Labs	C Labs	C1 Labs	Total	Grade
2+ to 4+, >50/oif, abundant gram positive bacilli, ± resembling *Coryne-bacterium* species/coryneforms/diphtheroids	65	5		6	76	4
3+ to 4+ gram positive coccobacilli	3	1			4	3
3+ gram positive bacilli, small, 4+ gram positive coccobacilli/ diphtheroids		1	3		4	3
4+ gram variable coccobacilli, snnp				1	1	3
4+ diphtheroids	2				2	1
4+, >25/oif gram positive bacilli, (± suggestive of coryneforms/ diphtheroids), 1+ to 4+, gram positive cocci	9	4	1	6	20	1
1+ to 4+ gram positive cocci/diplococci +/- refer	2			2	4	1
2+, 30-40/oif gram positive bacilli, ,<1/oif - 1+, 4+ gram negative bacilli +/- 5-10/oif gram positive cocci		1		1	2	0
4+ gram positive bacilli, 1+ yeast, snnp			1		1	0
1+ gram positive cocci, 1+ gram negative bacilli, snnp				1	1	0
2+, 3+ gram negative bacilli				2	2	0
snnp ± refer			2		2	ungraded
Wrong identifier	1	2	1	1	5	0
no report		1	1	2	4	0
Total	**82**	**15**	**9**	**22**	128	

2.4 Image Enhancement

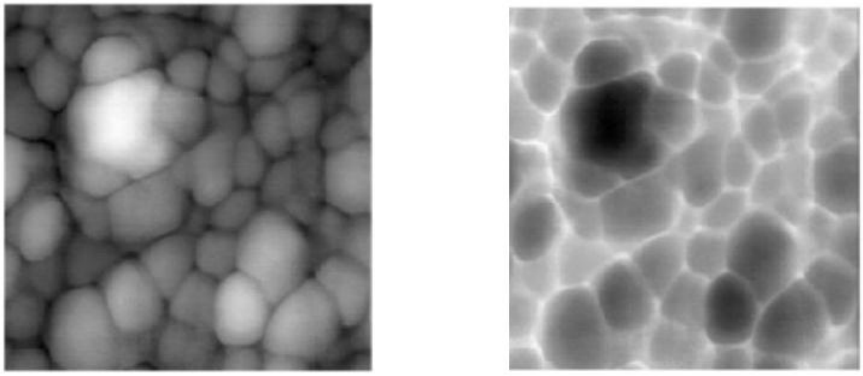
The main purpose of image enhancement is the process of manipulating an image so that the result is more suitable than original image for specific purposes [4][10]. In image enhancement, filtering method is used to enhance desire (structure) information and to suppress undesired (noise) object. Filtering operation is classified into two categories which are enhancing (high-pass filter), wherein desire object is

enhanced hopefully without affecting undesired object, and suppressing (low-pass filter), wherein undesired object is suppressed hopefully without affecting desire object [5].

There are the flows of method used in order to enhance the image:

i. Highlight fine details using Laplacian
ii. Enhance prominent edges using gradient
iii. Mask the Laplacian image using smoothed version of gradient image
iv. Increase the dynamic range of the intensity levels by using an intensity transformation

Thus, the processes image can be easily examined and interpreted. Another purpose of image enhancement is to facilitate printing of images or to allow automatic methods to perform measurements [11]. The result of image enhancement will improve the clarity of images for human viewing. There are many examples of enhancement operations such as removing blurring and noise, increasing contrast and revealing details [12]. Figure 2.9 shows the image after using image enhancement method.



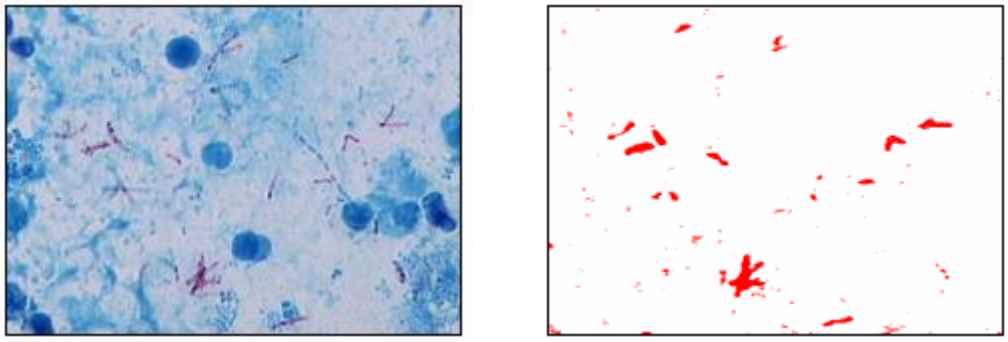
(a) (b)

Figure 2.9 (a) Original image (b) Enhance image

2.5 Image Segmentation by using Colour Thresholding

Segmentation process subdivides an image into its constituent regions or objects. The level of subdivision depends on the problem being solved, where the

segmentation should stop when the objects of interest in an application have been isolated. Image segmentation algorithms generally are based on one of the two basic properties of intensity values such as discontinuity and similarity [6]. Thresholding is a method of similarity category. It partitions an image into regions that are similar according to a set of predefined criteria. One simple way to accomplish Thresholding is by defining a range of brightness value in the original image, then the pixels are selected within the range as belonging to the foreground and all of other pixels are rejected to the background [7]. Figure 2.10 shows the image after use colour thresholding method.

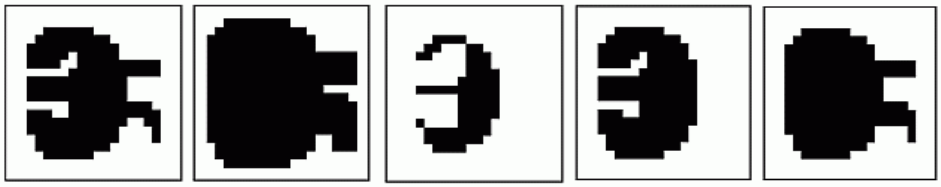


(a) (b)

Figure 2.10 (a) Original image (b) Threshold image

2.6 Morphological Image Processing

To identify the objects within an image is a very difficult task. Therefore, there is one way to simplify the problem which is to change the grayscale image into binary image. This way means that in which each pixel is restricted to a value of either 0 or 1. The morphological image processing is the one technique used on the binary image. Morphological operations have four basics used in the processing of binary images such as dilation, erosion, opening and closing [3]. The example of the techniques in morphological process by using Figure 2.11(a) as an original image as shown in Figure 2.11.



(a) (b) (c) (d) (e)

Figure 2.11 (a) Original image (b) Dilation image (c) Erosion image (d) Opening image (e) Closing image

2.6.1 Dilation Technique

In dilation image as shown in Figure 2.11(b), every background pixel that is touching an object pixel is changed into an object pixel. This technique makes the objects larger and can merge multiple objects into one.

2.6.2 Erosion Technique

In erosion image as shown in Figure 2.11(c), every object pixel that is touching a background pixel is changed into a background pixel. This technique makes the objects smaller and can break a single object into multiple objects.

2.6.3 Opening Technique

Opening is defined as an erosion technique followed by a dilation technique. As illustrated by Figure 2.11(d), opening removes small islands and thin filaments of object pixels.

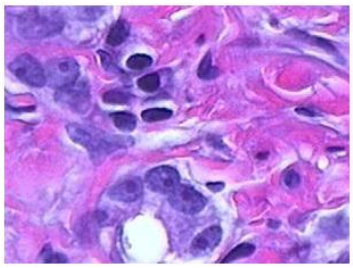
2.6.4 Closing Technique

Closing is defined as a dilation technique followed by an erosion technique. As illustrated by Figure 11(e), closing removes islands and thin filaments of background pixels.

These techniques are useful for handling noisy images where some pixels have the wrong binary value. For instance, it might be known that an object cannot contain hole, or that the object's border must be smooth [3].

2.7 K-Means Clustering

Cluster analysis is a way to organize and represent complex data sets. It is used routinely for data analysis in fields such as bioinformatics. The K-Means problem is to partition data into *k* groups such that the sum squared Euclidean distances to each mean is minimized [9][14][15]. K-Means is an algorithm to classify or to group the objects based on attributes or features into *k* number of group which is *k* is a positive integer number. The grouping is done by minimizing the sum of squares of distances between data and the corresponding cluster centroid. Thus, the purpose of K-Mean Clustering is to classify the data [13]. There is an example for K-Means Clustering method as shown in Figure 2.12.



(a)